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Immunoprecipitation of antigen-associated [^{32}P]-labelled nucleic acids from Crohn's disease mesenteric lymph nodes

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Summary. The methods of immunoselection and electrophoretic analysis of [^{32}P]-labelled nucleic acids have been applied to the problem of defining Crohn's disease (CD) specific antigen associated DNA or RNA, with the intention of identifying a presumptive aetiological microbial agent. Mesenteric lymph node derived cells from CD and control gastrointestinal disease cases were cultured *in vitro* with [^{32}P] orthophosphate after mitogenic stimulation with phytohaemaglutinin and pokeweed mitogen. Total cell lysates were immunoprecipitated with CD and control serum IgG fractions and immune complexes recovered with pansorbin. Antigen associated [^{32}P]-labelled nucleic acids were phenol/chloroform extracted and analysed by electrophoresis on polyacrylamide and agarose gels. No immunoprecipitated nucleic acid specific to CD tissues could be detected and no differences in antigen recognition between CD and control serum IgG were observed. No evidence was obtained for nucleic acid containing antigens either of the autoimmune type or of possible viral or microbial origin in CD mesenteric lymph nodes.

Keywords: Crohn's disease, immunoprecipitation, nucleic acids, antigen

Crohn's disease (CD) is a chronic granulomatous inflammation of the intestines in man of unknown aetiology but in which genetic, immunological and environmental factors appear to be involved (for reviews, see Farmer 1980; Brooke & Wilkinson 1980; Pena *et al.* 1981; Kirsner & Shorter 1982; Strickland & Jewell 1983). An infectious agent as the initiating cause of CD has long been suspected, although substantial microbiological research has failed to consistently isolate specific viral or bacterial pathogens from CD tissues (Whorwell 1981; Philpotts *et al.* 1979; 1980). Animal transmission experiments in which infectivity passed a 0.2 μm filter (Mitchell & Rees 1979), elevated titres of interferon (Bass *et al.* 1983) in CD and the presence of lymphocytotoxic and anti-double stranded RNA antibodies in CD patients and their household contacts (Korsmeyer *et al.* 1976) suggest a viral agent may be associated with the disease. Recently, Das *et al.* (1983) have reported the induction of lymphoma and hyperplastic lymph nodes in nude mice injected with 0.45 μm filtered CD mesenteric lymph node homogenates which, after 2–6 months, were immunoreactive against CD but not control sera (Das *et al.* 1983; Zuckerman *et al.* 1984). This sera reactivity was also observed in 30% of CD household contacts (Simon *et al.* 1984). Serum or IgG fractions from CD has also been

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shown to immunoprecipitate tissue specific proteins from CD colon homogenates but not control homogenates (Bagchi & Das 1984). The origin and nature of these CD-specific immunoreactive proteins and the relationship to the CD reactive antigens in the CD injected nude mice lymph nodes are unknown. Pertinent to these findings, it would seem that clarification of the role for an infectious agent in CD would profit from a search for a serological response to a presumptive agent and a subsequent characterization of the immunoreactive antigen(s).

We have therefore investigated whether CD sera will immunoprecipitate a CD specific antigen from CD mesenteric lymph nodes. If such an antigen was of viral origin it may be associated with viral nucleic acid, detectable by radioactive labelling and gel electrophoresis.

The object of the present study was to identify by electrophoretic analysis the [^{32}P] orthophosphate labelled nucleic acid components of CD-associated antigens, immunoprecipitated from CD mesenteric lymph node derived cell cultures. Demonstration of CD-specific, antigen-associated nucleic acids would allow a detailed characterization of aetiologically significant agents in CD at the level of DNA or RNA.

Materials and methods

Mesenteric lymph nodes and sera samples. Mesenteric lymph nodes adjacent to disease tissues were removed under sterile conditions from 16 patients with CD undergoing surgical resection at St George's Hospital, London, and one patient with diverticulitis (control). Nodes were rapidly transported to the laboratory on ice, minced with surgical blades to release the cells, which were then resuspended in RPMI 1640 (Dutch modified) culture medium (Flow Laboratories) supplemented with 100 units/ml of penicillin and streptomycin. Cells were filtered through fine gauze to remove tissue debris, washed twice, and resuspended in culture medium at 1×10^8 cells/ml. All procedures were performed under sterile conditions.

Sera samples were obtained from four normal healthy volunteers, two cases of appendicitis, one CD patient in remission and nine cases of CD with active disease undergoing surgical resection at St George's Hospital, London. Sera from three cases of systemic lupus erythematosus (SLE) were obtained from Dr R. Bernstein (Royal Postgraduate Medical School, London) and had been identified as precipitating the autoantigens Ro, Sm and RNP by counter-immunoelectrophoresis. Sera samples were stored at -70°C. Serum immunoglobulin G (IgG) was purified from whole serum, dialysed against 0.02 M Na_2HPO_4, pH 8, 0.02% azide, by chromatography on DEAE-Affi-Gel Blue (Bio-Rad Laboratories Ltd, England). Serum IgG so prepared has minimal ribonuclease and protease contamination (Bruck *et al.* 1982).

Preparation of [^{32}P]-labelled antigen. Freshly prepared mesenteric lymph node derived cells or pooled (four people) normal human leucocytes separated from whole blood on lymphocyte preparation media (Flow Laboratories) were washed three times and finally resuspended at 2×10^6 cells/ml in RPMI 1640 (Dutch modified) complete culture media supplemented with 100 units/ml of penicillin and streptomycin and 10% foetal bovine serum (FCS). A total of 2×10^8 cells for each antigen preparation was cultured for 18 h at 37°C in air in the presence of 4 μg/ml of phytohaemaglutinin (Reagent grade; Wellcome Laboratories) and 2.5 μg/ml of pokeweed mitogen (Sigma) to stimulate T-cell and B-cell lymphoblast formation, respectively. Viable cells were enumerated by vital dye exclusion. Cells were then washed once and resuspended at 1×10^7 viable cells/ml in a modified culture medium based on RPMI 1640 (Dutch modified) containing no phosphate salts and supplemented with 10% dialysed FCS. Cells were incubated at 37°C in air for 36 h in the presence of 20 μCi/ml of [^{32}P] orthophosphate (10 mCi/ml carrier

free; Amersham). Cells were harvested and washed free of [^{32}P] orthophosphate three times in equal volumes of phosphate containing culture media. All manipulations were performed under sterile conditions. Cells were then resuspended at 2×10^7 cells/ml in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA (NET buffer) containing 1 mM spermidine and 2 mM phenylmethylsulphonylfluoride as inhibitors of ribonucleases and proteases respectively, and chilled on ice. Cell lysis was achieved by sonication at 0°C for 2×20 s at 100 W (Braunsonic 1510; Melsungen AG) and monitored microscopically. The lysate was clarified by centrifugation at 15 000 *g* for 10 min at 4°C and the total cell extract supernatant stored at −70°C and used as the antigen source for immunoprecipitation.

In two separate labelling experiments, 2×10^8 lymph node derived cells from four and eight different CD patients respectively, were co-cultured as above and used as a source of mixed CD antigens. These cells prepared from individual patients had been stored frozen under liquid nitrogen in culture media containing 30% FCS and 10% DMSO. Before use cells were thawed rapidly at 37°C, resuspended and washed three times in 10 volumes of fresh medium with 20% FCS and finally combined with equal numbers of cells from the other CD patients at 2×10^6 viable cells/ml for culture and antigen preparation.

Immunoprecipitation of [^{32}P]-labelled antigen. Immunoprecipitations were performed as described by Lerner & Steitz (1979). Serum IgG (200 μg) in 15 μl NET buffer was incubated at 0°C for 2 h with 150–200 μl of [^{32}P]-labelled cell extract which had previously been pre-absorbed at 0°C for 20 min with 20 μl of pansorbin (Calbiochem). Before use, pansorbin was washed in NET buffer, 1·0% BSA to saturate nonspecific protein binding sites and was removed by centrifugation at 10 000 rpm for 3 min (MSE, microfuge). After incubation of IgG with cell extracts, 150 μl of pre-washed pansorbin was added and the IgG-antigen-pansorbin complexes were recovered after 1 h at 0°C by centrifugation. Pansorbin pellets were extensively washed five times with 1 ml of NET buffer containing 0.2% NP-40, 0.1% SDS and 0.5% Triton X-100, resuspended in 400 μl NET buffer, extracted with 400 μl phenol at 65°C for 15 min followed by phenol/chloroform/isoamyl alcohol (25:24:1) and re-extracted twice with chloroform at 20°C. The [^{32}P]-labelled nucleic acids in the aqueous phase were ethanol precipitated from 0.2 M sodium acetate, pH 5.5, at −20°C overnight, washed in 70% ethanol, redissolved in 50 mM Tris-HCL, pH 7.5, 1 mM EDTA, and stored at −70°C for electrophoretic analysis. Radioactivity was measured by liquid scintillation spectroscopy of trichloroacetic acid precipitable cell extracts on glass fibre GF/C filters or by Cerenkov counting for immunoprecipitated nucleic acids.

Electrophoretic analysis of immunoprecipitated [^{32}P]-labelled nucleic acids. Immunoprecipitated nucleic acids were analysed by electrophoresis in two gel systems in order to maximise the resolution of a complete size range from 40 to 60 000 bases. Small nucleic acids up to 800 bases were analysed on denaturing 8% polyacrylamide gels containing 7 M urea, 90 mM Tris-borate, pH 8.3, 1 mM EDTA for 3 h at 200 V after boiling in 10 M urea in the same buffer for 10 min. Large nucleic acids were denatured in 1 M glyoxal, 50% DMSO, 10 mM Na_2HPO_4, pH 7.0, at 50°C for 1 h and electrophoresed on 1% or 1.4% agarose gels in 10 mM Na_2HPO_4, pH 7.0, at 45 V for 3 h with buffer recirculation. The [^{32}P]-labelled nucleic acids were visualised in the fixed and dried gels by autoradiography for varying lengths of time, from 5–36 days. Aliquots of [^{32}P]-labelled whole cells and cell lysate supernatants before and after incubation with IgG/pansorbin were phenol/chloroform extracted in 1% SDS, NET buffer and the nucleic acids used as standards and to assess the structural integrity of the antigen-associated nucleic acid at different stages of the experiment. Ribonuclease free conditions were maintained

throughout these procedures using autoclaved buffers, acid washed and autoclaved glass and plastic ware.

Results

[^{32}P] labelling of CD lymph node cultures and immunoprecipitation

[^{32}P] Orthophosphate labelled antigen preparations were prepared from a total of seven cultures of 2×10^8 mesenteric lymph node derived cells (six CD and one diverticulitis case) and two cultures of normal pooled human peripheral blood leucocytes. Incorporation of [^{32}P] orthophosphate into these cells after 36 h ranged from $2.9–9.7 \times 10^9$/2×10^8 cells (mean: 4.22×10^9), equivalent to a mean of 4.22×10^6 ct/min/ml of cell extract. Two of the CD cultures were a mixture of mesenteric lymph node derived cells from in one case four and in another eight different patients, the cells having been stored frozen before use. All other cultures used fresh cells in monoculture. No differences between mixed and monocultures or between CD and control cultures were observed either in the incorporation of [^{32}P] or gross microscopical appearance. Aliquots of total cell extracts from each individual culture were incubated separately with 200 μg of 10 CD and six non-CD IgG fractions and the immune complexes formed were immunoprecipitated with pansorbin. After extensive washing, the nucleic acid components of the precipitated antigen were released and extracted with phenol/chloroform and recovered by ethanol precipitation. An average of 4.77×10^5 ct/min of [^{32}P]-labelled cell extract was incubated with each IgG fraction (range: $2.9–9.0 \times 10^5$). Approximately 0.5% (CD) and 0.54% (control) of total counts offered was recovered after immunoprecipitation and phenol extraction (range: 1380–3670, $n=72$, and 1807–4029, $n=40$, ct/min/incubation, respectively). SLE sera precipitated 4.1% of counts offered (range: 2.1%–4.6%, $n=8$). Small differences in the amount of radioactivity recovered between individual IgG fractions were most likely due to minor variations in the washing procedures.

Characterisation of the specificity of immunoprecipitation and structural integrity of the antigen source

In order to monitor the structural integrity of intracellular nucleic acid containing complexes, during the preparation of the cell extracts and immunoprecipitation procedures, the pattern of cytoplasmic ribosomal 28 S and 18 S RNA on glyoxal-agarose gels was examined. Intact total cellular RNA was prepared from pelleted whole cells cultured with [^{32}P] orthophosphate, by phenol/chloroform extraction after lysis in 1% SDS in NET buffer. No difference in the relative intensity of 18 S and 28 S RNA was seen between preparations of intact total RNA, cell extracts and cell extracts after incubation with serum IgG fractions and pansorbin (Fig. 1*a*). It was assumed, therefore, that any antigenic nucleoprotein particle (such as a virus) would remain intact under the conditions employed for its immunoselection.

Low molecular weight RNA species, immunoprecipitated from cell extracts by the previously defined SLE sera IgG, were recognised as components of the Ro, RNP and Sm autoantigens, since they precipitated correctly as specific subsets of small nuclear RNA (snRNA) as defined by Lerner *et al.* (1981) Fig. 2*a*, lanes 1 to 3. Defined SLE serotypes were used as a control in order to demonstrate that the immunoprecipitation method employed functioned specifically, and that ribonucleoprotein particles remained intact during these procedures. Also, the defined snRNA species were used as gel markers for comparison with any other immunoprecipitated nucleic acids.

Electrophoretic analysis of [^{32}P]-labelled nucleic acid components of immunoprecipitated antigens

High molecular weight antigen associated [^{32}P]-labelled nucleic acids immunoprecipi-

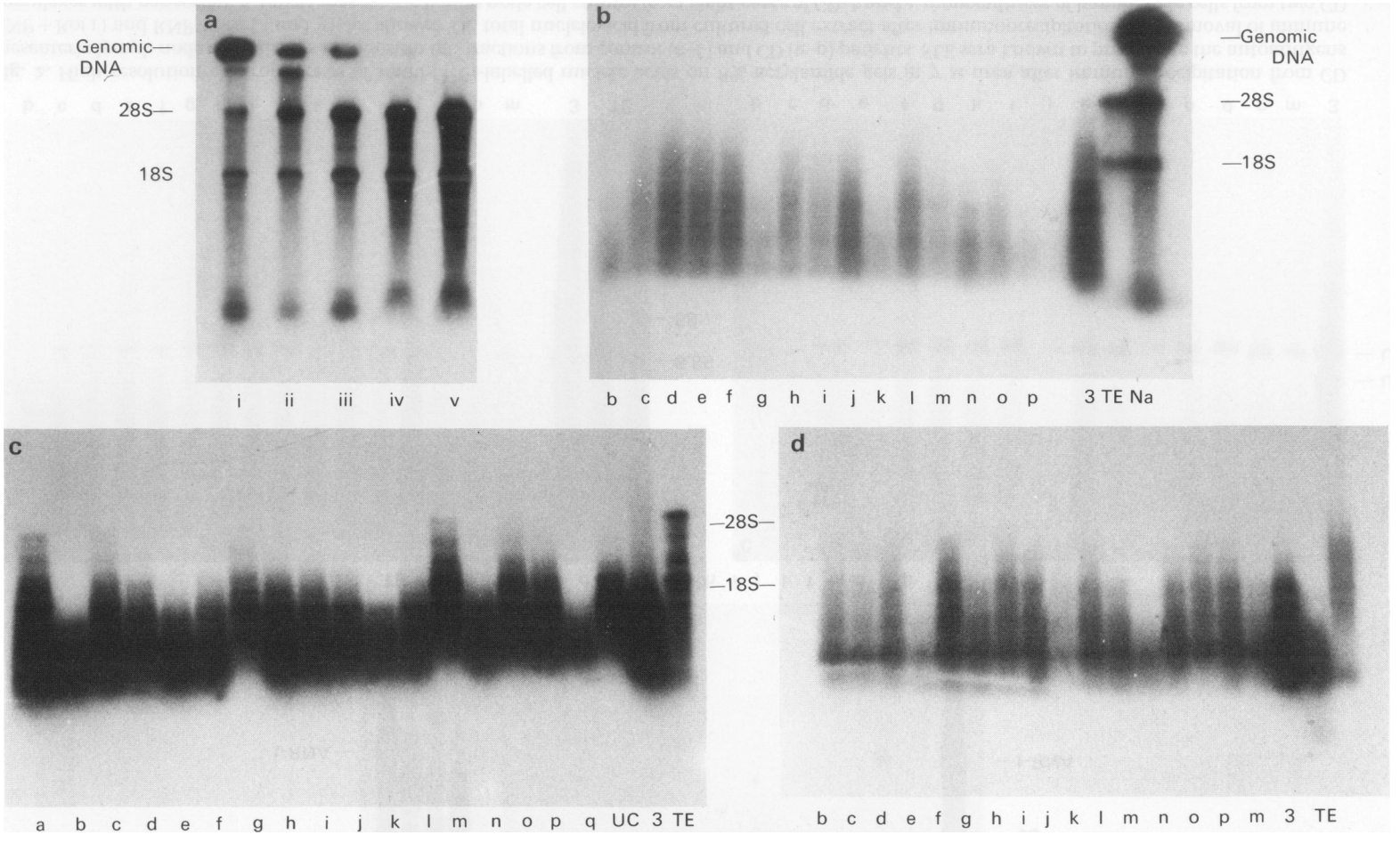

Fig. 1. Electrophoretic analysis of immunoprecipitated [^{32}P]-labelled nucleic acids from CD mesenteric lymph node cell cultures. a, Electrophoresis on a 1.4% agarose gel of total nucleic acid extracted from (i) whole cells after culture, (ii) cells after sonication, (iii) cell lysate after clarification by centrifugation and incubation at 0°C for 2 h, (iv) and (v) cell lysates from two separate cell cultures after incubation with IgG and removal of immune complexes with pansorbin. Ribosomal 18S and 28S RNA are indicated. *b*, *c* and *d*, electrophoresis on agarose gels of immunoprecipitated nucleic acids from a mixed mesenteric lymph node cell culture from eight CD patients (*b*) and two CD monocultures (*c* and *d*) using control (a–f), one ulcerative colitis (UC) and CD (g–q) serum IgG fractions after cell lysate and removal of immune complexes with pansorbin. TE, total nucleic acid from cultured cell extract after immunoprecipitation; Na, total nucleic acid extracted from whole cells. One SLE sera (3) known to precipitate RNP and Sm autoantigens is also shown. [^{32}P]-labelled nucleic acids were visualised by autoradiography.

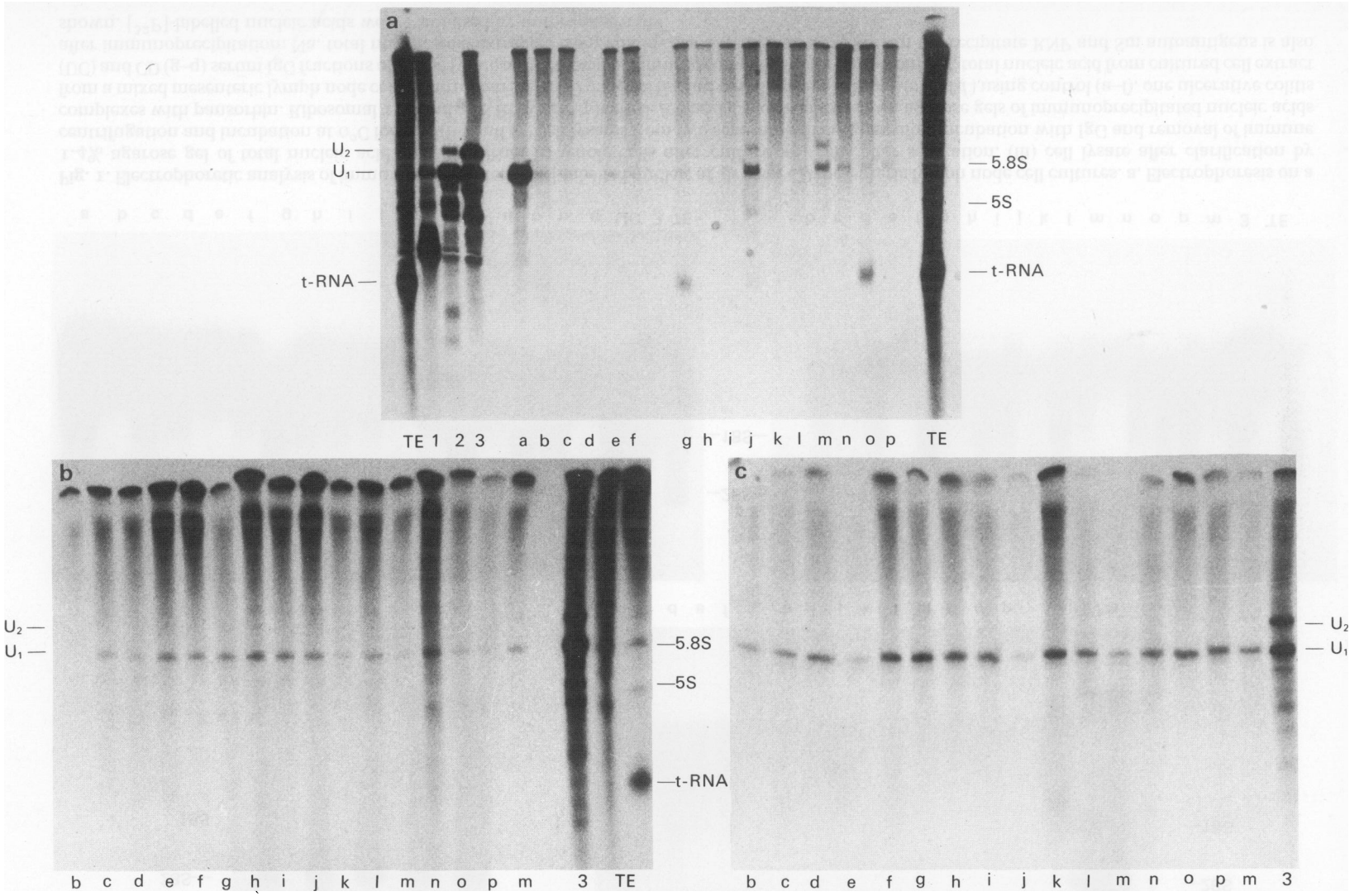

Fig. 2. High resolution electrophoresis of small [^{32}P]-labelled nucleic acids on 8% acrylamide gels in 7 M urea after immunoprecipitation from CD mesenteric lymph node cell cultures with serum IgG fractions from control (a–f) and CD (g–p) patients. SLE sera known to precipitate the autoantigens RNP+Ro(1) and RNP+Sm (2 and 3) are shown. TE, total nucleic acid from cultured cell extract after immunoprecipitation and removal of immune complexes with pansorbin; *a*, mixed mesenteric lymph node cell culture from eight cases of CD. *b* and *c*, monocultures of lymph node cells from two CD cases. Ribosomal 5.8S and 5 S RNA, transfer RNA (t-RNA) and the snRNA species U_1 and U_2 are indicated. [^{32}P]-Labelled nucleic acids were visualised

tated by IgG fractions of control (lanes a–f) and CD (lanes g–q) sera from CD and control antigen preparations have been analysed. The results from three CD (one mixed culture of cells from eight patients and two monocultures) antigen preparations are shown in Fig. 1. These results are representative of the pattern obtained from the remaining six other antigen sources. The corresponding low molecular weight nucleic acids are shown in Fig 2. Some low molecular weight nucleic acid was immunoprecipitated non-specifically from all antigen sources by CD and control IgG and migrated as a diffuse area at the bottom of the agarose gels. Higher resolution of this component on 8% polyacrylamide gels revealed one major band which comigrated with snRNA U_1. This band appeared in increased amounts from one of the appendicitis control IgG fractions (Fig. 2*a*, lane a). No other detectable nucleic acids were specifically precipitated either with CD or control IgG, regardless of antigen source. A band in the region of t-RNA was occasionally observed with four CD IgG (e.g. Fig. 2*a*, lane g,o), from three different CD and one control antigen sources. This band, however, appeared to be artifactual since it could not be demonstrated after repeated immunoprecipitations and probably resulted from incomplete washing of the pansorbin pellet. No evidence of a discrete high molecular weight nucleic acid component was obtained from any of the experiments, even after prolonged autoradiography for up to 36 days. No differences were observed between CD and control IgG fractions, although some differences between individual samples were seen (Fig. 1).

The results demonstrate that CD specific antigen associated nucleic acids cannot be detected by electrophoretic analysis after immunoprecipitation with CD serum IgG from [^{32}P]-labelled CD mesenteric lymph node cell extracts.

Discussion

We have applied sensitive methods of immunoselection and electrophoretic analysis of [^{32}P]-labelled nucleic acids to the problem of defining CD specific antigen associated nucleic acids with the intention of identifying a presumptive aetiological agent in CD. The results presented here do not provide any evidence for CD-specific nucleic acid containing antigens either of the autoimmune type or of possible viral or microbial origin. The absence of autoantibodies in CD, reactive with nucleic acid containing particles from mesenteric lymph nodes, confirms our previous observation that autoimmune reactions of the types seen in SLE, rheumatoid arthritis or myositis do not occur in CD (McFadden *et al.* 1985). We have assumed that since CD specific protein antigens have been previously detected by immunoprecipitation from seven out of eight CD intestinal extracts with 60% of CD sera used (Bagchi & Das 1984), then five out of our nine sera from active disease should be similarly reactive to CD tissue antigen. Yet none of the sera precipitated any CD specific nucleic acid from any of the six cultures from a total of 16 individual cases of CD. This indicates either that the previously defined CD gut antigens are not present in CD mesenteric lymph nodes, that they do not contain nucleic acid or that they are present at low titre or in small numbers of lymph node cell subpopulations, beyond detection by the methods employed in this study.

We conclude that antigen associated nucleic acids of viruses, or other microbial agents, could not be isolated from CD mesenteric lymph nodes with CD sera by immunoprecipitation. However, this does not exclude a role for an infectious (viral) agent in CD. Several models for the pathogenesis of CD may be proposed and in this study we assumed that the presumptive agent would be persistent in mesenteric lymph nodes and ellicit a continuing host antibody response. Immunofluorescence studies with CD sera on lymph nodes from nude mice injected with CD mesenteric lymph nodes (Das *et al.* 1983) supports this model. However, other mechanisms such as a transient infection produc-

ing primary damage and subsequent access to secondary pathogens, or a defective host immune response or immune evasion by the agent could account for the negative results presented here. Other reasons for the absence of CD specific nucleic acid might include the dissociation of the antigenic component from its nucleic acid moiety during cell lysate preparations; this might be the case with bacterial or mycobacterial agents but probably not with virus particles. Alternatively, the selected nucleic acid might have been too big for analysis on the agarose gel system, with a limit of detection up to about 60 000 bases. Investigation of viruses with larger genomes, such as from the Herpes virus family (including EBV, and cytomegalovirus), will require further separate experiments designed exclusively for their detection.

The potential for the investigation of an unknown aetiological agent in CD at the level of DNA or RNA is considerable. Previously applied DNA hybridisation techniques capable of detecting specific single copy sequences per cell could not detect sequences homologous with five species of adenovirus (Roche *et al.* 1981*a*) in CD tissues and although cytomegalovirus sequences could be detected, they were shown not to be disease specific (Roche *et al.* 1981*b*). The identification of a CD immunoreactive and antigen-associated DNA or RNA and its use in this kind of hybridisation analysis would be an important alternative to using probes from known viruses or bacteria of unspecified or indirect relevance to CD. Further studies using intestinal tissue as the antigen source or enriching for subsets of cells from gut or lymph node tissue for which the presumptive agent maybe trophic (Das *et al.* 1984) may yet identify a CD-specific nucleic acid of aetiological significance.

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